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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>A61K 37/00, 37/02, C07K 5/00</b> <b>C07K 7/00, 15/00, 17/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 93/15751</b> <b>(43) International Publication Date:</b> 19 August 1993 (19.08.93)
<b>(21) International Application Number:</b> PCT/US93/01263 <b>(22) International Filing Date:</b> 12 February 1993 (12.02.93)  <b>(30) Priority data:</b> 836,031 14 February 1992 (14.02.92) US 009,186 26 January 1993 (26.01.93) US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 836,031 (CIP) Filed on 14 February 1992 (14.02.92)  <b>(71) Applicant (for all designated States except US):</b> MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).		<b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> LOMBARDO, Victoria, K. [US/US]; 412 Southwoods Road, Belle Mead, NJ 08502 (US). TOLMAN, Richard, L. [US/US]; 28 Upper Warren Way, Warren, NJ 07060 (US). MARBURG, Stephen [US/US]; 50 Concord Avenue, Metuchen, NJ 08840 (US).  <b>(74) Agent:</b> ROSE, David, L.; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).  <b>(81) Designated States:</b> AU, BB, BG, BR, CA, CZ, FI, HU, JP, KR, LK, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> CHIMERIC TOXINS BINDING TO THE GnRH RECEPTOR  <b>(57) Abstract</b> <p>Analogs of GnRH are functionalized with unique linking groups so that they may be coupled to a cell-killing molecule. The chimeric toxin comprises a GnRH analog, a linking group and a toxin component which are administered to male and female animals where it is transported to organs containing cells with GnRH receptors such as pituitary glands in order to reduce secretions of sex steroids which results in sterility or in the reduction of tumors that require sex steroids for growth. The instant compounds are used as sterilizing agents and anticancer agents. The GnRH derivatives modified with the instant linking groups provides an advantage over prior chimera prepared by conjugation in that upon amino acid analysis of the conjugate, the modified GnRH derivative releases an unnatural amino acid which is readily quantified thus revealing the degree of conjugation between the GnRH analog and the toxin.</p>		

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TITLE OF THE INVENTION

CHIMERIC TOXINS BINDING TO THE GnRH RECEPTOR

CROSS REFERENCE TO RELATED APPLICATIONS

15

This application is a continuation-in-part of our copending application Serial No. 07/836031 filed 14 February 1992.

BACKGROUND OF THE INVENTION

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Considerable interest exists with respect to the subject of sterilization of animals. This is especially true of those concerned with veterinary medicine and animal husbandry, particularly as they relate to the subject of sterilization of domestic animals such as dogs, cats, cattle, sheep, horses, pigs, and the like. Various methods have been developed over the years to accomplish sterilization. For example, with respect to male cattle, the most widely used procedure for eliminating problems of sexual or aggressive behavior is sterilization through surgical castration. This is done in various ways, e.g., crushing the spermat

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cord, retaining the testes in the inguinal ring, or use of a rubber band, placed around the neck of the scrotum, to cause sloughing off of the scrotum and testes. However, most of these "mechanical"

5 castration methods have proven to be undesirable in one respect or another; for example they (1) are traumatic, (2) introduce the danger of anesthesia, (3) are apt to produce infection, and (4) require trained personnel. Moreover, all such mechanical  
10 castration methods result in complete abolition of the testes and this of course implies complete removal of the anabolic effects of any steroids which are produced by the testes and which act as stimuli to growth and protein deposition.

15 These drawbacks have caused consideration of various alternative sterilization techniques such as the use of chemical sterilization agents. However, the use of chemical sterilization agents has its own set of advantages and disadvantages. On the positive  
20 side, chemical sterilization eliminates the stress and danger associated with mechanical castration. Chemical sterilization also has the added advantage of allowing for retention of certain anabolic effects resulting from a continued presence of low levels of  
25 circulating testosterone. This is especially valuable in the case of animals raised for human consumption since circulating testosterone promotes growth, efficiency of feed conversion and protein deposition. Unfortunately, there are several  
30 disadvantages associated with chemical sterilization. For example chemical sterilization is often temporary rather than permanent; it also

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sometimes produces extremely severe, and even fatal, side effects.

In WO 9009799 to Nett certain GnRH analogs are coupled to a variety of toxins through an optional linking group consisting of 2-iminothiolane, SPDP (N-succinimidyl-3-(2-pyridyldithio/propionate), bis-diazabenzidine and glutaraldehyde. The compounds are disclosed as sterilizing agents.

In addition, Myers et al Biochemical Journal 227:1 pg 343 (1985) discloses a conjugate of a GnRH analog and the diphtheria A chain and pokeweed antiviral toxins coupled through SPDP.

Since toxins commonly have multiple ligand attachment sites, (generally amino or carboxy groups) and the linkage chemistry is not selective with regard to the number of GnRH molecules activated, there is no chemical control of the degree of conjugation. That is, conjugation can occur to one or through multiple attachments. By conventional chemistry, determination of the number of attachments of ligands is difficult or impossible. An unverified assumption of these prior works is that all toxin conjugates, regardless of their stiochiometry, are efficacious or equally efficacious.

Thus, while it is known generally that a toxin can be coupled to a GnRH analog through a linking group, the instant linking groups are unique and have been found to offer unique advantages in the efficacy of the final toxin conjugate, in the process for the manufacture thereof and in the analysis of conjugated peptide and toxin.

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In addition, various constructs of bacterial or plant toxins have been prepared in attempts to specifically target the toxin to certain cells or organs.

5           U.S. patent 4,545,985 teaches that *Pseudomonas* exotoxin A can be chemically conjugated to an antibody or to epidermal growth factor. While this patent further teaches that these conjugates can be used to kill human tumor cells, these chemically  
10 linked toxins have been shown to have undesirable, nonspecific levels of activity.

U.S. patent 5,036,047 teaches that free LHRH (alternate nomenclature for GnRH) can be administered  
15 along with an immunogenic conjugate of LHRH and a specific nonapeptide or decapeptide. The administration is reported to induce reversible sterilization.

20           U.S. patent 4,664,911 teaches that antibodies can be conjugated to the A chain or the B chain of ricin which is a toxin obtained from plants. Patent 4,664,911 further teaches that these conjugates  
25 can be used to kill human tumor cells.

U.S. patent 4,675,382 teaches that hormones such as melanocyte stimulating hormone (MSH) can be linked to a portion of the diphtheria toxin protein via peptide bonds. Patent 4,675,382 further teaches  
30 that the genes which encode these proteins can be joined together to direct the synthesis of a hybrid



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fusion protein using recombinant DNA techniques.  
This fusion protein has the ability to bind to cells  
that possess MSH receptors.

5                   Murphy, et al., PNAS USA 83:8258-8262  
(1986). This article teaches that a hybrid fusion  
protein produced in bacteria using recombinant DNA  
technology and consisting of a portion of the  
10                   diphtheria toxin protein joined to  
alpha-melanocyte-stimulating hormone will bind to and  
kill human melanoma cells.

Allured, et al., PNAS USA 83:1320-1324  
(1986). This article teaches the three dimensional  
15                   structure of the Pseudomonas exotoxin A protein.

Hwang, et al., Cell 48:129-136 (1987). This  
article teaches that the Pseudomonas exotoxin A  
protein can be divided into three distinct functional  
20                   domains responsible for: binding to mammalian cells,  
translocating the toxin protein across lysosomal  
membranes, and ADP ribosylating elongation factor 2  
inside mammalian cells. This article further teaches  
that these functional domains correspond to distinct  
25                   regions of the Pseudomonas exotoxin A protein.

Chaudhary, et al., PNAS USA 84:4538-4542  
(1987). This article teaches that hybrid fusion  
proteins formed between PE-40 and transforming growth  
30                   factor-alpha and produced in bacteria using  
recombinant DNA techniques will bind to and kill  
human tumor cells possessing epidermal growth factor  
receptors.

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European patent application 0 261 671, published 30 March 1988, teaches that a portion of the Pseudomonas exotoxin A protein can be produced which lacks the cellular binding function of the whole Pseudomonas exotoxin A protein but possesses the translocating and ADP ribosylating functions of the whole Pseudomonas exotoxin A protein. The portion of the Pseudomonas exotoxin A protein that retains the translocating and ADP ribosylating functions of the whole Pseudomonas exotoxin A protein is called Pseudomonas exotoxin - 40 or PE-40. PE-40 consists of amino acid residues 252-613 of the whole Pseudomonas exotoxin A protein as defined in Gray, et al., PNAS USA 81:2645-2649 1984. This patent application further teaches that PE-40 can be linked to transforming growth factor-alpha to form a hybrid fusion protein produced in bacteria using recombinant DNA techniques.

Kelley, et al., PNAS USA 85:3980-3984 (1988). This article teaches that a hybrid fusion protein produced in bacteria using recombinant DNA technology and consisting of a portion of the diphtheria toxin protein joined to interleukin 2 functions in mice to suppress cell mediated immunity.

Bailon, Biotechnology, pp. 1326-1329 Nov. (1988). This article teaches that hybrid fusion proteins formed between PE-40 and interleukin 2 and produced in bacteria using recombinant DNA techniques will bind to and kill human cell lines possessing interleukin 2 receptors.

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5           Edwards, et al., Mol. Cell. Biol. 9:  
2860-2867 (1989) describe the preparation of the modified TGF-alpha - PE<sub>40</sub> hybrid molecules that have been found to have utility in treating bladder tumor cells.

10           Heimbrook, et al., Proc. Natl. Acad. Sci. USA 87: 4697-4701 (1990) describe the in vivo efficacy of modified TGF-alpha - PE<sub>40</sub> in significantly prolonging the survival of mice containing human tumor cell xenografts.

15           A term for the selective delivery of chemotherapeutic agents to specific cell populations is "targeting". Drug targeting to specific cells can be accomplished in several ways. One method relies on the presence of specific receptor molecules found on the surface of cells. Other molecules, referred to as "targeting agents", can recognize and bind to these cell surface receptors. These "targeting agents" include, e.g., antibodies, growth factors, or hormones. "Targeting agents" which recognize and bind to specific cell surface receptors are said to target the cells which possess those receptors. For example, pituitary cells that release lutenizing hormone (LH) possess a protein on their surfaces that recognizes and binds with GnRH. GnRH is therefore, a  
20           "targeting agent" for these cells.

25           "Targeting agents" by themselves do not kill cells. Other molecules including cellular poisons or toxins can be linked to "targeting agents" to create hybrid molecules that possess both cell  
30           targeting and cellular toxin domains. These hybrid

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5 molecules function as cell selective poisons by virtue of their abilities to target selective cells and then kill those cells via their toxin component. Some of the most potent cellular poisons used in constructing these hybrid molecules are bacterial toxins that inhibit protein synthesis in mammalian cells. Pseudomonas exotoxin A is one of these bacterial toxins, and has been used to construct hybrid "targeting - toxin" molecules (U.S. Patent 10 4,545,985).

Pseudomonas exotoxin A intoxicates mammalian cells by first binding to the cell's surface, then entering the cell cytoplasm and inactivating elongation factor 2 which is a cellular protein required for protein synthesis. Pseudomonas exotoxin A has been used to construct anticancer hybrid molecules using monoclonal antibodies and protein hormones. However, one problem with these hybrid molecules is that they exhibit toxicity towards normal cells. At 15 least part of the toxicity associated with hybrid molecules containing Pseudomonas exotoxin A is due to the ability of Pseudomonas exotoxin A by itself to bind to and enter many types of mammalian cells. Therefore, hybrid molecules formed between Pseudo- 20 monas exotoxin A and specific "targeting agents" can bind to many normal cells in addition to the cells recognized by the "targeting agent". One method of dealing with this problem is to modify Pseudomonas exotoxin A so that it is no longer capable of binding 25 to normal cells. This can be accomplished by removing that portion of the Pseudomonas exotoxin A molecule 30

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which is responsible for its cellular binding activity. A truncated form of the Pseudomonas exotoxin A molecule has been prepared which retains the ability to inactivate elongation factor 2 but no longer is capable of binding to mammalian cells. This modified Pseudomonas exotoxin A molecule is called Pseudomonas exotoxin - 40 or PE<sub>40</sub> (Hwang, et al., Cell 48:129-136 1987).

The instant invention utilizes various constructs of GnRH, a linking group and a toxin. However, unlike the prior art constructs which rely upon the techniques of biotechnology and recombinant DNA, the instant invention describes the preparation of site-specific toxin constructs using the techniques of synthetic organic chemistry.

In addition, the prior art constructs were difficult to analyze for amino acid content because there was no way of knowing how many of the GnRH peptides bonded with the toxin since the toxins, being polypeptides; generally had more than one site with which the GnRH, or othe peptide, could bond. The instant linking groups, upon degradation, release a specific marker in the form of an unnatural amino acid. The amount of this marker allows the facile calculation of the exact ratio of peptide groups to toxins.

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SUMMARY OF THE INVENTION

This invention is concerned with an agent which is selectively toxic to the LH releasing cells of the pituitary. This selective toxicity is  
5 achieved by first removing, from a general toxin, the domain involved in binding to cells which results in greatly reduced general toxicity. This binding domain is replaced, in our invention, with a group (GnRH) which specifically binds to LH releasing cells  
10 and thus creates a very specific, new binding domain. The conjugation of these two moieties is accomplished with specific and improved linking groups that also aid in the analysis of the peptide to toxin ratio. This new, chimeric conjugate is now  
15 selectively toxic to those cells in the pituitary gland which release luteinizing hormone (LH). The death of these cells and the concomitant inability to generate LH results in the irreversible sterilization of the animal. While a broad category of toxic  
20 agents can be utilized in the chimeric toxin conjugate, conjugates based on Pseudomonas exotoxin A are preferred.

DESCRIPTION OF THE INVENTION

25 The chimeric toxic agents are best described in the following structural representation:

Q-Ser-Tyr-W-X-Arg-Y-Z

|  
L1

|  
L2

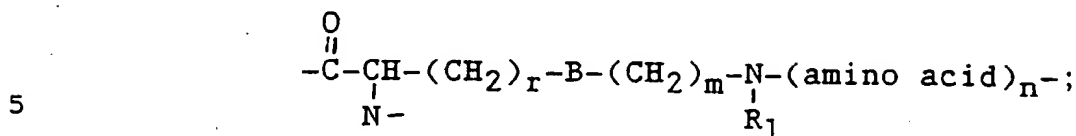
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T

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where Q is PyroGlu-His-Trp, N-acetyl-4-Cl-Phe<sup>1,2</sup>-Trp or 3-indolylpropionyl;

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W is the D or L amino acid with a pendant linking functionality such as

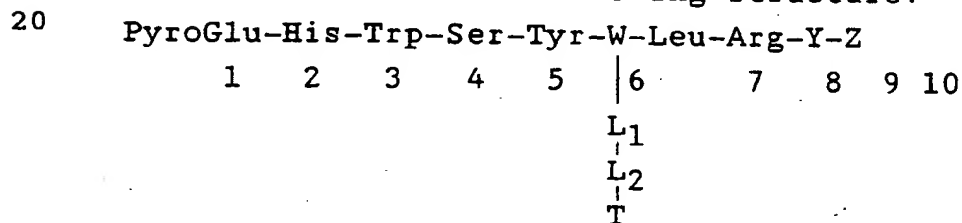


where

- r is 1 or 2;  
 m is 1 to 4;  
 n is 0 or 1;  
 B is CH<sub>2</sub>, O, S or N; and  
 R<sub>1</sub> is hydrogen, C<sub>1</sub>-C<sub>6</sub>alkyl or C<sub>3</sub>-C<sub>8</sub>cycloalkyl;  
 X is Leu or Nle;  
 Y is Pro or 4-hydroxy-Pro; and  
 Z is Gly, NH<sub>2</sub>, D-Ala-NH<sub>2</sub>, NH-Et, NH-Pr or Aza-Gly-NH<sub>2</sub>.

L<sub>1</sub>, L<sub>2</sub> and T are as defined below.

Preferred compounds of the instant invention are realized in the following structure:



where the decapeptide is GnRH with the normal 6-position amino acid (W=Gly) deleted and replaced by W=D-Lys or D-Orn. The use of D-Lys<sup>6</sup>-GnRH is a preferred targeting agent with which to bind the toxic construct to the LH releasing pituitary cells, it will be recognized that variations of D-Lys<sup>6</sup>-GnRH

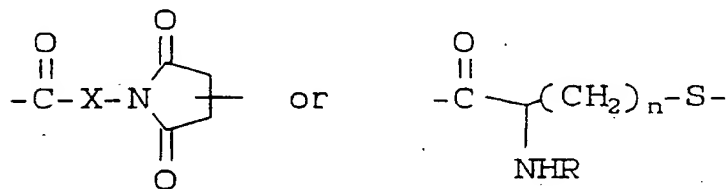
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that will still bind to the GnRH receptor of the pituitary will be useful in this invention. All that is required is that the 6-position amino acid possess an amino group for binding to the linking group and that the remainder of the peptide bind to the GnRH receptor on the pituitary gland cells.

Z in the above formula is Gly-NH<sub>2</sub>, ethylamide, or Arg-Gly NH<sub>2</sub>;

Y is Pro or 4-hydroxy-Pro;

L<sub>1</sub> and L<sub>2</sub> are independently



where X is C<sub>1</sub>-C<sub>5</sub> alkylene, phenyl or C<sub>5</sub>-C<sub>6</sub> cycloalkylene;

R is C<sub>1</sub>-C<sub>3</sub> alkanoyl;

n is 1 or 2; and

T is a toxin group; provided that the carbonyl ends of each of L<sub>1</sub> and L<sub>2</sub> are bonded to either the GnRH derivative or to the toxin.

It will be appreciated by those skilled in the art that 4-hydroxy-Pro can exist as D and L isomers and as cis and trans isomers. All such isomers, and the racemic mixture of the D and L isomers are intended to be included in this invention.



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The toxin can be any toxin that is capable of destroying the LH releasing cells of the pituitary, referred to as gonadotrophs. The toxins can be plant derived toxins, bacteria derived toxins or chemical toxins. Examples of plant derived toxins are ricin, modeccin, abrin, pokeweed antiviral protein,  $\alpha$ -amanatin, gelonin ribosome inhibiting protein, (RIP) or RIP derived from wheat, corn, rye, flax and the like. Examples of bacteria derived toxins are diphtheria toxin, Pseudomonas exotoxin, shiga toxin and the like. Examples of chemical toxins are melphalan, methotrexate, nitrogen mustard, doxorubicin, daunomycin and the like.

The preferred toxins are those derived from Pseudomonas exotoxin. The most preferred toxins are those segments of Pseudomonas exotoxin wherein the binding domain has been deleted or partially deleted so that the toxin retains its potential for cell toxicity but that the toxin lacks the ability to bind to animal cells, except when coupled with the GnRH targeting agent.

One example of such a Pseudomonas toxin has had amino acids 1-252 deleted, which comprises most or all of the binding region and retaining amino acids 253-613 which contain the cell translocation region and the toxin region. This Pseudomonas exotoxin fragment has been identified as PE-40 - See Hwang *et al.*, supra, Kondo *et al* *J. Biol Chem* 263 pg 9470-9475 (1988), Chaudharg *et al*, DNAS-USA, 87 pg 308-312 (1990) and US Patent 4892827 to Pastan *et al*.

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The *Pseudomonas* exotoxin fragment PE-40 has been further modified by removing additional amino acids 365-380 and further providing the point mutations of deleting one or more of the Lys amino acids and replacing these with a different amino acid that will not bond with L<sub>1</sub> or L<sub>2</sub>. Since, however, Lys is the amino acid that is bonded to the L<sub>1</sub> or L<sub>2</sub> at least one Lys is preferred to be retained in the *Pseudomonas* exotoxin peptide fragments. One such modified PE-40 has been designated PE-38M and it is shown in Table 1 where the numbers above the peptide sequence refer to the *Pseudomonas* exotoxin sequence.

The various *Pseudomonas* exotoxin fragments are prepared using the techniques of biotechnology and recombinant DNA. However, once the *Pseudomonas* exotoxin has been prepared, it is bonded to the linking groups L<sub>1</sub> and L<sub>2</sub> and the D-Lys<sup>6</sup>-GnRH using synthetic organic chemical techniques.

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## Sequence Listing Table PE-38M

253 300  
 5 MLQGTKLMAEEGGS LAALTAHQACHLPLETFTTRHRQPRGWEQLEQCGYPVQRLVALYLAAR  
 350 364  
 LSWNQVDQVIRNALASPGSGDLGEAIREQPEQARLALT LAAAESERFVRQGTGNDEAGAAN  
 381 400  
 GPADSGDALLERNYPTGA EFLGDDVSFSTRGTQNWTVRLLQAH RQLEERGYV FVG YHGTF L  
 450 500  
 10 EAAQSIVFGGVRARSQDLDAIWRGFYIAGDPALAYGYAQDQEPDARGRIRNGALLRVYVPRSSL  
 550  
 PGFYRTSLTLAAPEAAGEVERLIGHPLPLRLDAITGP EEEGGRLETILGWPLAERTVVIPSAIP  
 600 613  
 15 TDP RNVGGDLDPSSIPDQEQAISALPDYASQPGQPPREDLR

Additional toxins are \_\_\_\_\_ from the  
 Pseudomonas exotoxins by the selection \_\_\_\_\_ of the  
 amino sequence such as ....

20 DESCRIPTION OF THE DRAWINGS

Figure 1 is a mass spectrometric analysis of  
 the toxin conjugate of DLs<sup>6</sup>-GnRH coupled to PE38 with  
 from 1 to 5 GnRH moieties per toxin. The six  
 25 membered peaks show an increasing molecular weight  
 order, the unconjugated protein and the protein  
 conjugated sequentially with from 1 to 5 GnRH  
 analogs. The analysis was carried out using Laser  
 Time-of-flight (TOF) technique or a matrix consisted  
 30 matrix description TOF mass spectrometer.

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Figure 2 is a mass specromative analysis of the PE38 starting material of approximately 38K molecular weight. The two smaller peaks represent doubly or tripled charged molecules.

5        Figures 3, 4 and 5 are SDS-PAGE Gel electrophoreis of the conjugates of PE38 D-Lys<sup>6</sup> GnRH (Figures 3 and 4) or PE38M and D-Lys<sup>6</sup>-GnRH (Figure 5). The preparation of the instant toxin conjugates is shown in Reaction Scheme 1 using D-Lys<sup>6</sup> GnRH and  
10      PE-38M for exemplification.

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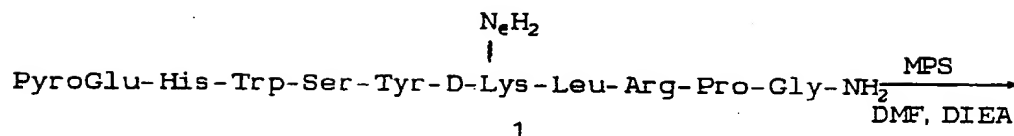
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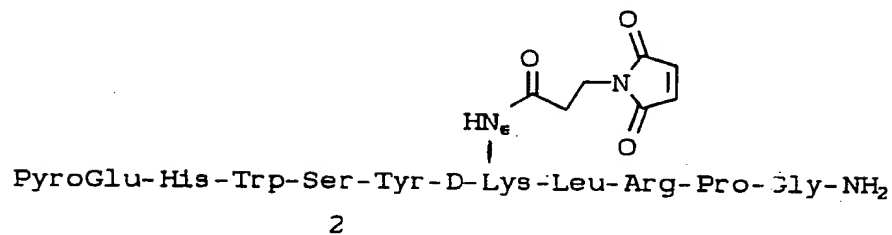
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REACTION SCHEME 1

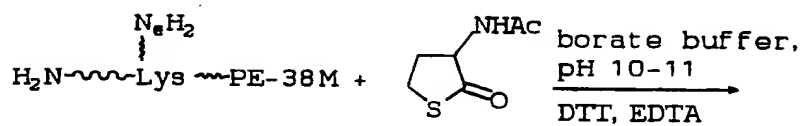
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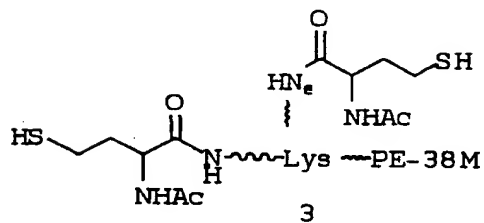
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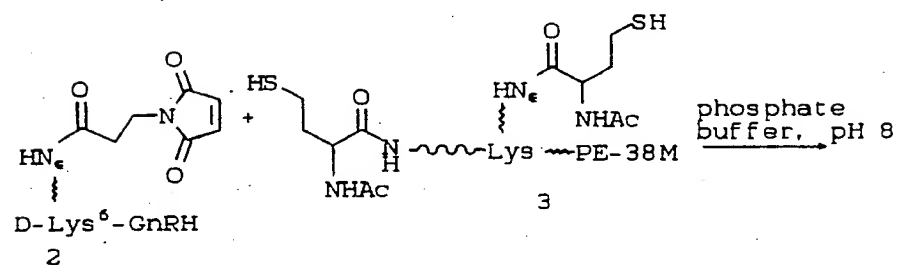
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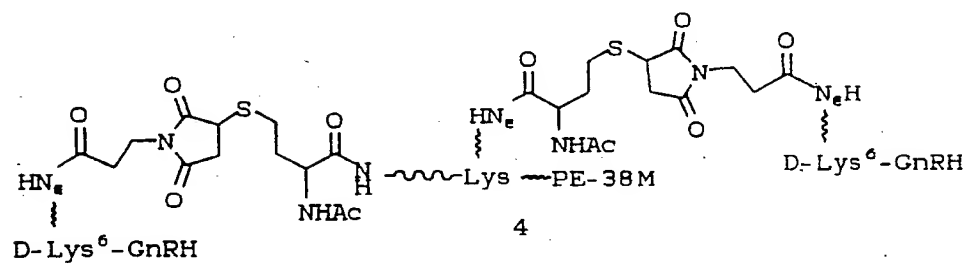
REACTION SCHEME I (CONT'D)

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In this reaction sequence the GnRH is first modified with one of the L<sub>1</sub> and L<sub>2</sub> linking groups and the modified Pseudomonas exotoxin peptide is modified with the other of the L<sub>1</sub> and L<sub>2</sub> linking groups. The L<sub>1</sub> and L<sub>2</sub> linking groups are attached to all free or unprotected primary amines on the GnRH derivative and the Pseudomonas exotoxin. In the case of the D-Lys<sup>6</sup>GnRH the linking group is bonded to the amine at the end of the alkyl chain of the Lys. Since the N-terminus of the D-Lys<sup>6</sup>GnRH is a pyroglutamyl, no reaction can occur since no free primary amine is present. In the case of the Pseudomonas exotoxin, reaction can occur at any Lys present. The PE-38M modified protein has only one Lys but other modified Pseudomonas exotoxins may have more than one. In addition, the N-terminus of the Pseudomonas exotoxin is a free amine which is available for reaction with L<sub>1</sub> and L<sub>2</sub>. If no other Lys were present on the modified Pseudomonas exotoxin, coupling with the D-Lys<sup>6</sup>-GnRH would still occur since the N-terminus amine would still provide the linking site. With more than one amine, multiple couplings of the D-Lys<sup>6</sup>-GnRH will occur.

In Reaction Scheme 1 the first step is the reaction of D-Lys<sup>6</sup>-GnRH with L<sub>1</sub> which for purposes of illustration is shown as the maleimidoyl alkanoyl group. The other linking group, N-alkanoyl cysteine or N-alkanoyl homocysteine could also be used. The D-Lys<sup>6</sup>-GnRH is prepared using known peptide synthesis techniques, preferably the solid phase peptide synthesis.

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The reaction for the preparation of the  $L_1$ -D-Lys<sup>6</sup>-GnRH is carried out using an active ester of the maleimidoyl alkanoyl group. Preferred esters are the esters made from maleimidoyl alkanoyl acid and N-hydroxy succinimide, pentafluorophenol or p-nitro phenol. The ester with N-hydroxy succinimide is most preferred. The reaction is carried out in a polar solvent with a base selected from either (a) a non-nucleophilic organic base such as N,N-diisopropyl ethylamine or (b) a weak inorganic base such as sodium or potassium carbonate. The polar solvent can be N,N-dimethylformamide, water, acetonitrile or mixture thereof. N,N-Dimethylformamide is preferred. The reaction is carried out at from 0 to 25°C, preferably at room temperature and is generally complete in from 10 to 90 minutes. The work-up of the reaction is to initially neutralize the base present with an acid such as trifluoroacetic acid, and the pH of the mixture is brought to about 2-4. The product is then isolated using techniques known to those skilled in the art.

In the next step of the reaction scheme, the Pseudomonas exotoxin is reacted with the other linking group  $L_2$  at any and all of the unprotected primary amines. For purposes of illustration the Pseudomonas exotoxin is shown as PE-38M which has two primary amines available for reaction, the N-terminus amine and the epsilon amine or the Lys, shown as  $N_\epsilon$ . With such an arrangement two equivalents of the  $L_1$ -D-Lys<sup>6</sup>-GnRH will react with each equivalent of the PE-38M.



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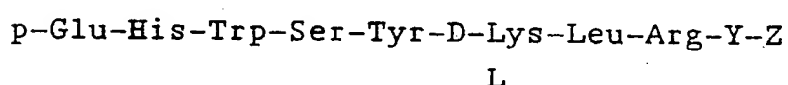
The reaction is carried out in an aqueous buffer which provides for a pH of greater than 10. Borate buffer solution with a pH of about 11 is preferred. Included in the reaction mixture preferably is dithiothreitol and/or the disodium salt of ethylenediaminetetraacetic acid. These reagents are generally added in considerable excess in order to prevent the reactive mercapto group from forming a disulfide bond with like groups. For the same reason, the reaction is carried with the strict exclusion of oxygen, generally by using a nitrogen atmosphere. The reaction is carried out at from 0 to 25°C, preferably room temperature, and is generally complete in from 5 to 18 hours. Before the L<sub>2</sub>-Pseudomonas exotoxin can be conjugated with the L<sub>1</sub>-D-Lys<sup>6</sup>-GnRH, the dithiothreitol, the ethylenediaminetetraacetic acid disodium salt and any hydrolyzed N-alkanoyl homocysteine thiolactone must be removed. Thus the reaction mixture is purified of such reagents prior to the next step. The most convenient method for doing so is to dialyze the reaction mixture. The dialysis solution free of extraneous reagents is used in the next step without further treatment.

The final step is the coupling of the L<sub>1</sub> D-Lys<sup>6</sup>GnRH with the L<sub>2</sub>-Pseudomonas exotoxin which is carried out under nitrogen at a pH of from 8 to 10 with a excess of the L<sub>1</sub> D-Lys<sup>6</sup>GnRH. Generally from 2 to 20 equivalents of the GnRH reagent are used for each equivalent of the L<sub>1</sub> substituent on the Pseudomonas exotoxin. The reaction is generally very fast and is complete in just 1-5 minutes although further aging of up to 2 hours has not been found to

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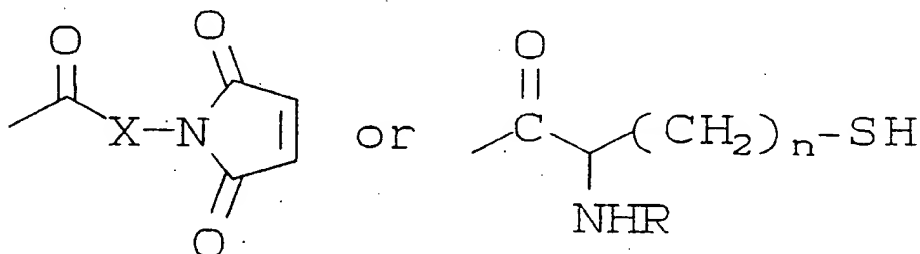
be detrimental. The coupled D-Lys<sup>6</sup>GnRH L<sub>1</sub>-L<sub>2</sub>-pseudo-monas exotoxin is isolated using techniques known to those skilled in the art. It has been found that dialysis of the reaction mixture is a convenient  
 5 method for the removal of unwanted products. Since the conjugated product will generally be administered by injection, the resultant dialysis solution may be sterile filtered and used directly for percutaneous administration.

10 The intermediate compounds 2 and 3 are important aspects of the instant invention and represent novel compounds. The novel intermediates are realized in the following structural formulae:



and L-T

20 where L is independently



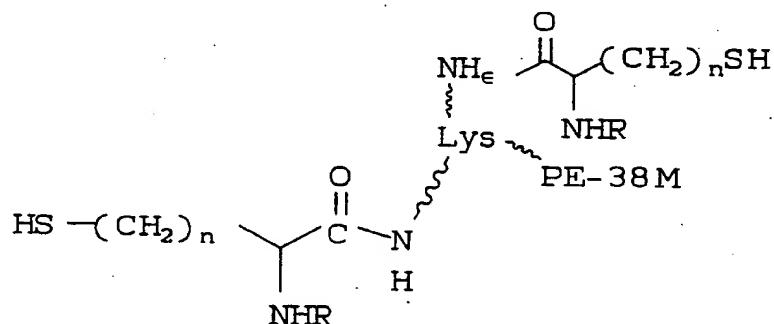
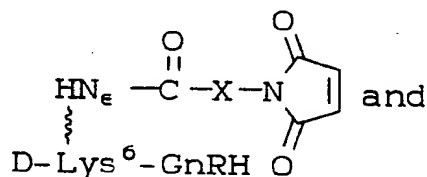
where

30 X is C<sub>1</sub>-C<sub>5</sub>alkylene, phenyl or C<sub>5</sub>-C<sub>6</sub>cycloalkylene;  
 R is C<sub>1</sub>-C<sub>3</sub>alkanoyl;  
 n is 1 or 2; and  
 T is a toxin.

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The linking groups, L, are bonded to the available primary amines of the D-Lys<sup>6</sup>-GnRH and the toxin, preferably Pseudomonas exotoxin A or fragment thereof. Where, as in the Pseudomonas exotoxin, more than one primary amine is available, more than one L group will be reacted therewith.

While either value of L may be bound to either the D-Lys<sup>6</sup>-GnRH or the toxin, it is preferred to bond the maleimido alkanoyl group to the GnRH and the N-alkanoyl homocysteinyll group to the toxin. The most preferred toxin is PE-38M and the most preferred GnRH derivative is D-Lys<sup>6</sup>-GnRH, thus, the most preferred intermediates of the instant invention are:



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The instant site-specific toxins which connect a GnRH analog with a toxin through a unique linking group offer significant advantages in the preparation and analysis of the toxin conjugates. The toxin moieties often have more than one amine function which can be coupled to the GnRH analog through various linking groups (See Nett *et al*). However, in preparing such toxin conjugates, it is not certain if all of the amine functions, generally found on the amino acid lysine, will react with the GnRH linking group, thus at the completion of the reaction the conjugate will have to be analyzed to determine the number of GnRH moieties that have been bonded to the toxin. A further complication arises when, as often occurs, the resultant conjugate is actually a mixture of conjugates with differing numbers of GnRH analogs coupled to the toxin. The analysis of the toxin conjugates is usually done through amino acid analysis, however, since the GnRH and toxin all break down to normal amino acids, the determination of the number of GnRH moieties bonded to the toxin is a very long and tedious process.

The instant linking group, however, under amino acid analysis breaks down to beta-alanine, an unnatural amino acid. Thus during amino acid analysis, the ratio of lysine to beta-alanine is determined which reveals the extent of the conjugation. This is a much more direct and accurate method of determining the degree of conjugation which greatly facilitates the use of the toxin conjugates as chemical sterilization agents.

- 25 -

Applicants have found that the GnRH conjugates of the instant invention are particularly effective in causing the toxic compound T to be specifically targeted to the gonadotropin-secreting cells of the anterior pituitary gland. Indeed, they are the only cells to which the gonadotropin-releasing hormone portion of the conjugate will bind. Hence, these toxic compounds, bound to an analog of gonadotropin-releasing hormone, can be employed to permanently destroy a subpopulation of the anterior pituitary cells and thereby eliminate the gland's ability to secrete gonadotropins. This in turn causes the animal's gonads to atrophy and lose their ability to function for reproductive purposes. That is to say that, without functioning gonadotrophs, an animal is not able to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and thus is rendered sterile. Applicants have postulated that the compounds of this patent disclosure inhibit synthesis of LH, and presumably other proteins made by gonadotrophs, because they tend to inhibit all protein synthesis once these compounds gain entry into a cell. It should also again be noted that applicants' compounds allow "chemical castration" to be employed in place of surgical castration.

Thus, the use of these compounds has great utility in human medicine as well as in veterinary medicine. This follows from the fact that there are several important biological reasons for employing castration and antifertility drugs in humans. For example, breast and prostate cancers are but two

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examples of sex steroid-dependent tumors which respond to such hormonal manipulation. At present, the only reliable way to inhibit steroid-dependent tumor growth is through administration of counter-regulatory hormones (e.g., DES in prostate cancer),  
5 sex-steroid hormone binding inhibitors (e.g., tamoxifen in breast cancer) or surgical castration. Thus the potential medical uses of such chemical castration compounds are vast and varied. For  
10 example, prostate cancer remains an important cause of cancer deaths and represents the second leading cancer of males. The present palliative treatment for advanced prostate cancer cases involves reduction of serum testosterone/DHT levels through use of  
15 surgical castration. It should also be noted that for purposes of disease and/or fertility control, especially in humans, it may be desirable to use applicant's compounds to ablate pituitary gonadotrophs in conjunction with other modes of  
20 treatment. For example, it is anticipated that chronic administration of progestins and estrogens to females and androgens to males might be necessary to prevent loss of secondary sex characteristics, behavior and osteoporosis. However, through  
25 judicious use of the herein disclosed compounds, especially in combination with appropriately administered sex steroids, desirable antifertility effects can be achieved. Another area of application in human medicine is treatment of endometriosis.  
30 This condition, which produces painful growth of endometrial tissue in the female peritoneum and

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pelvis also responds to inhibition of sex steroid synthesis. Those skilled in this art will also appreciate that the herein disclosed compounds could be used to partially reduce sex-steroid secretions, and thus reduce or eliminate certain hormone related behavior problems while retaining improved growth stimulation.

The dose/time adjustments associated with the use of these compounds can vary considerably; however, these compounds are preferably administered by injection into a mammal in concentrations of from about 0.1 to about 10 milligrams per kilogram of the mammal's body weight. Sterilization may be accomplished with as few as one injection; but multiple treatments (e.g., based upon concentrations of from about 0.03 milligrams once every 4 days to about 1 milligram per kilogram of body weight for 20 days) are alternative sterilization schemes. Furthermore, as sterilization agents, the compounds of this patent disclosure can be used before or after puberty. They too are especially useful in those areas of animal husbandry where the anabolic benefits of non-surgical sterilization techniques can contribute to meat production and/or quality. In one preferred embodiment of this invention the compounds of this invention are administered to male cattle between the ages of about 8 weeks and 20 weeks at least once and in a concentration of from about 0.1 to about 10 milligrams per kilogram of the animal's body weight.

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The toxic moieties T of the herein disclosed compounds are obtainable from both natural and synthetic sources. For example, pokeweed antiviral protein can be isolated from leaves of pokeweed plants and purified by gel filtration chromatography. It can then be, by way of example, conjugated to D-Lys<sup>6</sup>-desGly<sup>10</sup>-Pro<sup>9</sup>GnRH-ethylamide via the amino group on the lysine and through a sulfhydryl group introduced into the pokeweed antiviral protein by the instant novel linking groups. In any event, one of the chief advantages of these compounds is their ability to produce permanent sterilization without strong toxic side effects. Hence these compounds may be used on mammals such as human beings, domestic animals, pets or wild animals. Moreover, they can be administered as a single injection which can induce permanent and irreversible sterility in both male and female mammals. However, an alternative approach to achieve sterilization is through multiple injections at lower dosages than those employed in a single treatment or by slow release implants (i.e., biodegradable formulations).

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EXAMPLE 1Procedure for the Preparation of a 6-D-Lys-GnRH/Lys-PE38M Congugate

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General: All reagents were used as received by the supplier. In the case of solvents, HPLC-grade was used where available. HPLC (binary gradient) was performed on a Waters 600E system with Waters 484 tunable U.V. detector (Aufs=0.1 analytical or 2.0 preparative scale) and recorded on a Waters 746 Data Module. A Waters WISP™712 autosampler (2000 µL sample loop) was used for analytical samples. A Rheodyne 7125 manual injection port (5000 µL sample loop) was used for preparative samples. A = H<sub>2</sub>O, 0.1% TFA; B=CH<sub>3</sub>CN, 0.1% TFA. Mass spectra were taken on a Finnegan MAT 90, spectrophotometer (positive ion, NBA matrix).

20

Abbreviations: Standard amino acid abbreviations are used. RT, room temperature; DCC, 1,3-dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; DIEA, N,N-diisopropylethylamine; MPS, β-maleimidopropionic acid N-hydroxy-succinimide ester; GnRH, gonadotropin releasing hormone; PBS, phosphate buffered saline; DTT, dithiothreitol; EDTA-2Na, ethylenediaminetetraacetic acid disodium salt; NBA, 3-nitro-benzyl alcohol.

30

6-D-Lys-GnRH, 1:PyroGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH<sub>2</sub>:

The peptide was synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (SPPS) using an ABI model 431A

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synthesizer and single couplings (DCC/HOBT). The peptide was cleaved (2 h, RT) from the resin using reagent R (1 mL/100 mg resin, TFA/thioanisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC (Waters PrepPak®25 x 10<sup>3</sup> C<sub>18</sub>; 10 mL/min; 10-20% B, 0-20 min.; then 20-35% B, 20-40 min.; λ=230 nm).

6-D-Lys-GnRH: FAB-MS (positive ion, NBA matrix) Calc. M+1 1254.44; Found M+1 = 1254.4

(N<sub>ε</sub>-maleimidopropanoyl)-6-D-Lys-GnRH, 2:PyroGlu-His-Trp-Ser-Tyr-(N<sub>ε</sub>-maleimidopropanoyl)-D-Lys-Leu-Arg-Pro-Gly-NH<sub>2</sub>:

6-D-Lys-GnRH (10 mmol, 12.5 mg) was dissolved in N,N-dimethylformamide (0.5 mL/mg) and DIEA (50 mmol, 9 μL) added. The mixture was stirred briefly (RT) and β-maleimidopropionic acid N-hydroxysuccinimide ester (MPS; 20 mmol, 5.2 mg) was introduced in one portion. After 30 min reaction time, 10 μL TFA was added to the reaction mixture and the solvent removed in vacuo. The peptide was purified by reverse phase HPLC (Waters PrepPak® 25 x 10<sup>3</sup> Delta-Pak™ C<sub>18</sub>; 10 mL/min; 10-25% B, 0-30 min.; then 25% B, 30-35 min; λ=230 nm).

(N<sub>ε</sub>-maleimidopropanoyl)-6-D-Lys-GnRH, FAB-MS (positive ion, NBA matrix) Calc. M+1: 1405.56; Found M+1=1405.6

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Conjugation of (N<sub>ε</sub>-maleimidopropanoyl)-6-D-Lys-GnRH, to Lys-PE38M, 4:

To a sterile 15 mL polyethylene centrifuge tube with septum was added Lys-PE38M (.221  $\mu$ mol, 3.0 mL, 2.8 mg/mL) in PBS. The pH of the solution was adjusted to 10.8 by the addition of 350  $\mu$ L of 1.0 M, pH 11.0 borate buffer. Dithiothreitol (11.0  $\mu$ mol, 1.7 mg) and ethylenediaminetetraacetic acid disodium salt (22.1  $\mu$ mol, 8.2 mg) were added and the protein mixture vortexed until all solids were in solution. N-Acetylhomocysteine thiolactone (22.1  $\mu$ mol, 3.5 mg) was introduced in one portion and the solution degassed and purged with N<sub>2</sub> (degas/purge repeated 5X). The mixture was aged in an N<sub>2</sub> box at RT for 6.5 h, then charged to Spectropor 2 dialysis tubing and dialyzed (RT) as follows: vs. 1) 4L degassed, N<sub>2</sub>-sparged, 0.1 M, pH 8.0 phosphate buffer which contained 10 mg EDTA-2 Na and 0.25 mg DTT (16 h); 2) 4L degassed, N<sub>2</sub>-sparged, 0.01 M, pH 8.0 phosphate buffer containing 10 mg EDTA-2 Na, 0.25 mg DTT (6h). The thiolated exotoxin was then transferred to a sterile 15 mL polyethylene centrifuge tube (3.90 mL). An Ellman assay on 325  $\mu$ L of this material indicated that a total of 0.350  $\mu$ mol of SH was present. To the remaining thiolated material (0.321 mmol SH, 3.57 mL solution) was added 2 (1.60  $\mu$ mol, 2.25 mg). The reaction was then vortexed briefly and aged in an N<sub>2</sub> box (RT, 1h). The toxin mixture was charged to Spectropor 2 dialysis tubing and dialyzed (4°C) as follows: vs.1) 4 L, 0.01 M, pH 7.0 phosphate buffer (18 h); 2) 4 L, 0.01 M, pH 7.0 phosphate buffer (46 h); and 3) 4 L deionized H<sub>2</sub>O (7h). The conjugate was centrifuged to pellet any unsuspended material

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and passed through a sterile filter (Millipore 0.22  $\mu$ m, Millex®-GV). This provided 4, which had HPLC characteristics that were distinct from unconjugated Lys-PE38M.

Lys-PE38M:RP-HPLC (250 mm x 4.6 mm Vydac C<sub>4</sub>; 1.5 mL/min.; 36-41% B, 0-30 min.;  $\lambda$ =215 nm): RT = 18.16 min.

## EXAMPLE 2

### Preparation of PE38M

Plasmid PJH4 (Ref. Hwang. J. Cell (1987, 48; 129-136) contains the coding sequence for PE<sub>1-613</sub>. Oligonucleotide directed mutagenesis as described in 15.51-15.73, Molecular Cloning, 2nd ed (1989) edited by Sambrook, Fritsch & Maniatis (Cold Spring Harbor Press) has been used as a convenient way to make deletions/mutations in the PE molecule. An NDE1/Hind III double digest is carried out on PJH4 resulting in linearization of the construct and clipping of a 12 b<sub>p</sub> segment which includes the ATG start codon of the PE coding sequence. Two complementary oligonucleotides are synthesized, annealed and ligated into the NDE1/Hind III splice site. The oligomers have the following nucleotide sequence: I - 5' TAT GCT GCA GGG TAC CAA GCT TAT GGC CGA AGA<sup>3</sup>' and II - 5' AGC TTC TTC GGC CAT AAG CTT GGT ACC CTG CAG CA3'. The modified PE insert has a sequence of MLQGTKLMAEE constructed at the N-terminus. This plasmid is designated PJH42.

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The plasmid PJH42 is partially cut with Ava I. The linear form of DNA is isolated, completely digested with Hind III, and the resulting 5.1 Kb fragment isolated. Sl nuclease treatment is carried out to allow blunt end ligation of the sticky ends and the plasmid is recircularized and designated PJH43. This results in a PE with deletion of amino acids 4-252.

A 553 bp Sal I/Bam HI fragment of plasmid PJH43 is cloned into M13 mp19. An oligonucleotide, 50 nucleotides in length with the structure 5' GGC GTC GCC GCT GTC CGC CGG GCC GTT GGC CGC GCC GGC CTC GTC GTT GC3', is synthesized and annealed to the single stranded M13 vector to facilitate (loop out) mutagenesis generating a deletion of amino acids 365-380 of the PE insert, resulting in the sequence:

..... AGAANGPADSGDALL.....  
 ↑↑  
 364 381

A 505 bp Sal I Bam HI fragment is excised from the replicative form of the mutant DNA in M13 and ligated with a 3.7 Kb Sal I Bam HI fragment of the plasmid PJH43. This new plasmid is designated PJH44.

A Bam HI/EcoR I fragment of 460 nucleotides is excised from PJH44 and cloned into M13 mp19. This fragment contains the nucleotide sequence for three lysines that are mutated at the carboxy end of the coding sequence: lysines 590, 606 are mutated to glutamines and lysine 613 is mutated to an arginine.

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Oligo directed mutations are then carried out successively at each of the lysines with the following oligomers:

5 Lysines 590-5' GCT GAT CGC CTG TTC TTG GTC GGG GAT  
GCT GGA C 3'

Lysines 606-5' GTC CTC GCG CGG CGG TTG GCC GGG CTG  
GCT G 3'

10 Lysines 613-5' CGG TCG CGG CAG TTA ACG CAG GTC CTC  
GCG CGG 3'

15 The Bam H1 EcoR 1 fragment is excised from the replicative form of the mutant DNA in M13 and ligated with a 3.4 Kb Bam H1/EcoR 1 fragment of the plasmid PJH44. The linearized plasmid is then recircularized, designated PJH45 and used for expression of the modified PE, identified as PE38m, from a commercially available strain of E. coli, HB 101, available from Bethesda Research Laboratories.

20

### EXAMPLE 3

#### Preparation of other GnRH Analogs

25 N-Ac-1,2-Di-p-Chloro-Phe-6-DLys-GnRH, Ac-4-C1-Phe-  
4-C1-Phe-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-Gly-NH<sub>2</sub>:

30 The peptide is synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (Fmoc chemistry) using an ABI model 431A synthesizer and double couplings (DCC/HOBT) for 4-C1-Phe and single couplings for the remaining residues. The amino terminus is capped by treatment

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with acetic anhydride (5-10 mL) until the resin beads give a negative Kaiser test for the presence of an amine (0.5-8 h). The peptide is cleaved (2 h-4 h, RT) from the resin using reagent R (0.5 mL-3 mL/100 mg resin, TFA/thioanisole/ethanedithiol/anisole, 90:5:3:2). The peptide is precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC and characterized by FAB-MS.

6-DLys-10-DAla-GnRH, H-Pgl-His-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-DAla-NH<sub>2</sub>:

The peptide was synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (Fmoc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The peptide was cleaved (3 h, RT) from the resin using reagent R (2.0 mL/100 mg resin, TFA/thioanisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC. Characterization by FAB-MS of 6-D-Lys-10-D-Ala-GnRH (positive ion, NBA matrix) Calc (m+1) = 1268.5; Found (m+1) = 1267.5.

6-DLys-9-Pro-NHEt-GnRH, H-Pgl-His-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-NHEt:

The peptide is synthesized on Oxime or Merrifield resin by solid phase peptide synthesis (Boc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The peptide is cleaved (2 h-72 h, RT) from the resin with anhydrous

- 36 -

ethyl amine. The crude protected peptide is precipitated with diethyl ether, collected by suction filtration, and dried overnight (over P<sub>2</sub>O<sub>5</sub>). The protecting groups are removed from the dry peptide by  
5 treatment with anhydrous HF (0°C, 0.5-2 h, 5-30 mL) in the presence of anisole (0.2-2 mL) and dimethyl phosphite (0.1-1 mL). The excess HF is removed in vacuo and the residue triturated with diethyl ether. The peptide is purified by preparative  
10 reverse phase HPLC and characterized by FAB-MS.

6-DOrn-GnRH, H-Pgl-His-Trp-Ser-Tyr-DOrn-Leu-Arg-Pro-Gly-NH<sub>2</sub>:

The peptide was synthesized on Rink amide  
15 MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (Fmoc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The peptide was cleaved (3 h, RT) from the resin using reagent R (2.0 mL/100 mg resin, TFA/thio-  
20 anisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC. Characterization by FAB-MS of 6-D-Orn-GnRH (positive ion, NBA matrix).  
25 Calc (m+1) 1239.4; Found (m+1) 1239.5.

3-Indolylpropionyl-6-DLys-GnRH, 3-Indolylpropionyl-Ser-Tyr-DLys-Leu-Arg-Pro-Gly-NH<sub>2</sub>:

The peptide was synthesized on Rink amide  
30 MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (Fmoc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT).



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The peptide was cleaved (3 h, RT) from the resin using reagent R (2.0 mL/100 mg resin, TFA/thio-anisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC. Characterization by FAB-MS of 3-indolylpropionyl-6-D-Lys-GnRH (positive ion, NBA matrix) Calc (m+1) 990.2; Found (m+1) 990.7.

3-Indolylpropionyl-6-DLys-9-Pro-NHEt-GnRH, 3-Indolylpropionyl-Ser-Tyr-DLys-Leu-Arg-Pro-NHEt:

The peptide is synthesized on Oxime or Merrifield resin by solid phase peptide synthesis (Boc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The 3-indolylpropionyl moiety is incorporated as N-formyl-3-indolepropionic acid. The peptide is cleaved (2 h-72 h, RT) from the resin with anhydrous ethyl amine. The crude protected peptide is precipitated with diethyl ether, collected by suction filtration, and dried overnight (over P<sub>2</sub>O<sub>5</sub>). The protecting groups are removed from the dry peptide by treatment with anhydrous HF (0°C, 0.5-2 h, 5-30 mL) in the presence of anisole (0.2-2 mL) and dimethyl phosphite (0.1-1 mL). The excess HF is removed in vacuo and the residue triturated with diethyl ether. The peptide is purified by preparative reverse phase HPLC and characterized by FAB-MS.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Lombardo, Victoria R  
Marburg, Steven  
Tolman, Richard L
- (ii) TITLE OF INVENTION: Chimeric Toxins Binding to the GnRH  
Receptor
- (iii) NUMBER OF SEQUENCES: 1
- 10 (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: David L. Rose  
(B) STREET: P.O. Box 2000  
(C) CITY: Rahway  
(D) STATE: New Jersey  
(E) COUNTRY: USA  
15 (F) ZIP: 07065
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- 20 (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: Rose, David L  
(B) REGISTRATION NUMBER: 26,332  
25 (C) REFERENCE/DOCKET NUMBER: 18652
- (ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: (908) 594-4777  
(B) TELEFAX: (908) 594-4720
- 30

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## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 355 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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 1 5 10 15

Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu Thr Phe Thr Arg  
 20 25 30

His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys Gly Tyr Pro  
 35 40 45

Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn  
 50 55 60

Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro Gly Ser Gly  
 65 70 75 80

Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg Leu  
 85 90 95

Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln Gly  
 100 105 110

Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Gly Pro Ala Asp Ser Gly  
 115 120 125

Asp Ala Leu Leu Glu Arg Asn Tyr Pro Thr Gly Ala Glu Phe Leu Gly  
 130 135 140

- 40 -

	Asp	Gly	Gly	Asp	Val	Ser	Phe	Ser	Thr	Arg	Gly	Thr	Gln	Asn	Trp	Thr	145	150	155	160
	Val	Glu	Arg	Leu	Leu	Gln	Ala	His	Arg	Gln	Leu	Glu	Glu	Arg	Gly	Tyr	165	170	175	
5	Val	Phe	Val	Gly	Tyr	His	Gly	Thr	Phe	Leu	Glu	Ala	Ala	Gln	Ser	Ile	180	185	190	
	Val	Phe	Gly	Gly	Val	Arg	Ala	Arg	Ser	Gln	Asp	Leu	Asp	Ala	Ile	Trp	195	200	205	
10	Arg	Gly	Phe	Tyr	Ile	Ala	Gly	Asp	Pro	Ala	Leu	Ala	Tyr	Gly	Tyr	Ala	210	215	220	
	Gln	Asp	Gln	Glu	Pro	Asp	Ala	Arg	Gly	Arg	Ile	Arg	Asn	Gly	Ala	Leu	225	230	235	240
	Leu	Arg	Val	Tyr	Val	Pro	Arg	Ser	Ser	Leu	Pro	Gly	Phe	Tyr	Arg	Thr	245	250	255	
15	Ser	Leu	Thr	Leu	Ala	Ala	Pro	Glu	Ala	Ala	Gly	Glu	Val	Glu	Arg	Leu	260	265	270	
	Ile	Gly	His	Pro	Leu	Pro	Leu	Arg	Leu	Asp	Ala	Ile	Thr	Gly	Pro	Glu	275	280	285	
20	Glu	Glu	Gly	Gly	Arg	Leu	Glu	Thr	Ile	Leu	Gly	Trp	Pro	Leu	Ala	Glu	290	295	300	
	Arg	Thr	Val	Val	Ile	Pro	Ser	Ala	Ile	Pro	Thr	Asp	Pro	Arg	Asn	Val	305	310	315	320
	Gly	Gly	Asp	Leu	Asp	Pro	Ser	Ser	Ile	Pro	Asp	Gln	Glu	Gln	Ala	Ile	325	330	335	
25	Ser	Ala	Leu	Pro	Asp	Tyr	Ala	Ser	Gln	Pro	Gly	Gln	Pro	Pro	Arg	Glu	340	345	350	
	Asp	Leu	Arg														355			

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WHAT IS CLAIMED IS:

1. A toxin conjugate having the formula:

Q-Ser-Tyr-W-X-Arg-Y-Z

L<sub>1</sub>

L<sub>2</sub>

T

where

Q is PyroGlu-His-Trp, N-acetyl-4-Cl-Phe<sup>1,2</sup>-Trp,  
or 3-indolylpropionyl;

W is the D or L amino acid

O

-C-CH-(CH<sub>2</sub>)<sub>r</sub>-B-(CH<sub>2</sub>)<sub>m</sub>-N-(amino acid)<sub>n</sub>-;  
NH R<sub>1</sub>

where

r is 1 or 2;

m is 1 to 4;

n is 0 or 1;

B is CH<sub>2</sub>, O, S or N; and

R<sub>1</sub> is hydrogen, C<sub>1</sub>-C<sub>6</sub>alkyl, or  
C<sub>3</sub>-C<sub>8</sub>cycloalkyl;

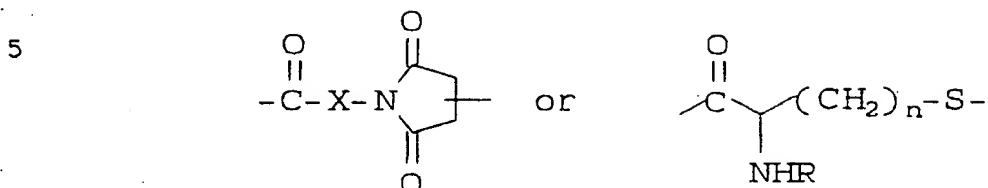
X is Leu or Nle;

Z is Gly-NH<sub>2</sub>, ethylamide or Aza-Gly-NH<sub>2</sub>;

Y is Pro or 4-hydroxy Pro;

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$L_1$  and  $L_2$  are independently



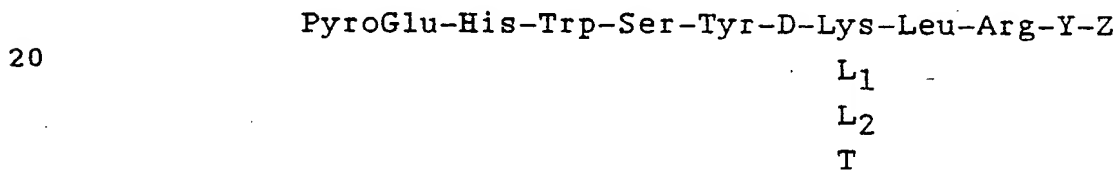
10 where X is  $\text{C}_1$ - $\text{C}_5$  alkylene, phenyl, or  $\text{C}_5$ - $\text{C}_6$  cycloalkylene;

R is  $\text{C}_1$ - $\text{C}_3$  alkanoyl; and

n is 1 or 2; and

15 T is a toxin group; provided that the carboxyl end of each of  $L_1$  and  $L_2$  are bonded to either the GnRH derivative or to the toxin.

2. A toxin conjugate of Claim 1 having the formula:



25 where Y, Z,  $L_1$ ,  $L_2$  and T are as defined Claim 1.

3. The toxin conjugate of Claim 2 where the toxin is capable of destroying the LH releasing cells of the pituitary gland.

30 4. The toxin conjugate of Claim 3 where the toxin is a plant derived toxin, a bacteria derived toxin or a chemical toxin.

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5           5.    The toxin conjugate of Claim 4 where  
the plant derived toxin is ricin, modeccin, abrin,  
pokeweed antiviral protein,  $\alpha$ -amantin, gelonin  
ribosome inhibiting protein (RIP) or RIP derived from  
wheat, corn, rye or flax.

10           6.    The toxin conjugate of Claim 4 where  
the chemical toxin is melphalan methotrexate,  
nitrogen mustard, doxorubicin or daunomycin.

15           7.    The toxin conjugate of Claim 4 where  
the bacteria derived toxin is diphtheria toxin,  
Pseudomonas exotoxin or shiga toxin.

20           8.    The toxin conjugate of Claim 7 where  
the bacteria derived toxin is Pseudomonas exotoxin or  
a segment thereof.

25           9.    The toxin conjugate of Claim 8 where  
the Pseudomonas exotoxin has been modified such that  
the binding domain has been partly or completely  
deleted.

30           10.   The toxin conjugate of Claim 9 where  
the Pseudomonas exotoxin has been modified to delete  
amino acids 1-252 and retain amino acid 253-613.

          11.   A process for the preparation of a  
compound of Claim 1 which comprises treating a GnRH  
derivative having the formula:

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Q-Ser-Tyr-W-X-Arg-Y-Z

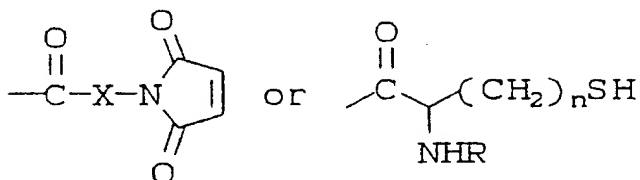
 $L_1'$ 

where Q, W, X, Y and Z are defined in Claim 1 with  
 5 toxin having the formula

 $L_2' - T$ 

where T is a toxin and  $L_1'$  and  $L_2'$  independently are

10



15

where X, R and n are as defined in Claim 1.

12. The process of Claim 11 where the GnRH  
 derivative has the formula:

PyroGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Y-Z

20

 $L_1'$ 

13. The process of Claim 12 where the GnRH  
 derivative is bonded to  $L_1'$  having the formula

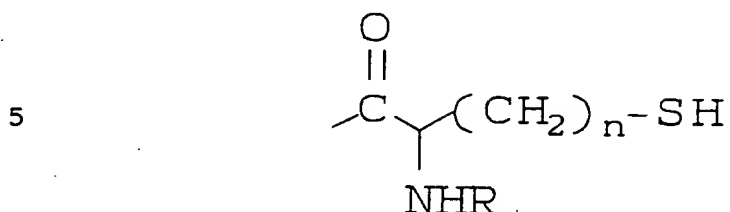
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- 45 -

and the toxin T is bonded to L<sub>2</sub>' having the formula



10 14. The process of Claim 13 where the GnRH derivative is D-Lys<sup>6</sup>-GnRH.

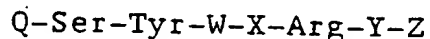
15 15. The process of Claim 13 where the toxin T is a Pseudomonas exotoxin.

16 16. The process of Claim 14 where the Pseudomonas exotoxin is PE-38M.

20 17. A method for the sterilization of animals which comprises administering to such animals an effective amount of a toxin conjugate of Claim 1.

25 18. A composition useful for the sterilization of animals which comprises an inert carrier and an effective amount of a toxin conjugate of Claim 1.

19. A GnRH derivative having the formula



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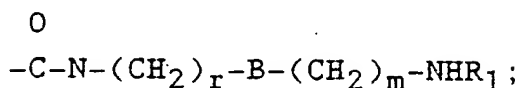
- 46 -

where

Q is PyroGlu-His-Trp-, N-acetyl-4-Cl-Phe<sup>1,2</sup>-Trp,  
or 3-indolylpropionyl;

W is the D or L amino acid

5



where

r is 1 or 2;

m is 1 to 4;

10

B is CH<sub>2</sub>, O, S or N; and

R<sub>1</sub> is hydrogen, C<sub>1</sub>-C<sub>6</sub>alkyl,  
C<sub>1</sub>-C<sub>6</sub>alkanoyl, C<sub>3</sub>-C<sub>8</sub>cycloalkyl or  
an amino acid linked through its  
C-terminus;

15

X is Leu or Nle;

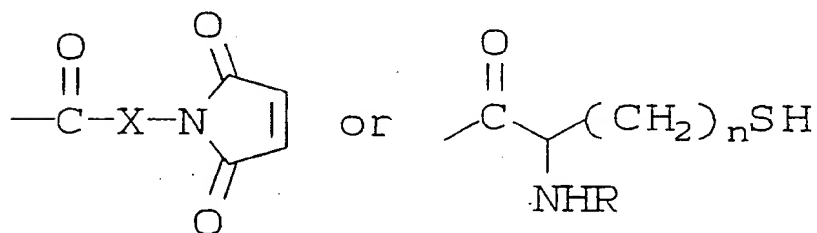
Y is Pro or 4-hydroxyPro;

Z is Gly-NH<sub>2</sub>, ethylamide or Aza-Gly-NH<sub>2</sub>; and

L is

20

25



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- 47 -

where

X is C<sub>1</sub>-C<sub>5</sub>-alkylene, phenyl or C<sub>5</sub>-C<sub>6</sub>  
cycloalkylene;

R is C<sub>1</sub>-C<sub>3</sub>-alkanoyl; and

5 n is 1 or 2.

20. A GnRH derivative of Claim 19 having  
the formula

PyroGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Y-Z

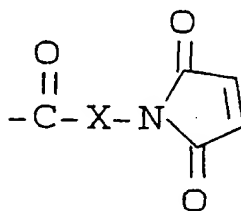
10

L

where Y, Z and L are as defined in Claim 19.

21. A compound of Claim 19 where Y is Pro  
and L is

15



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and X is as defined in Claim 19.

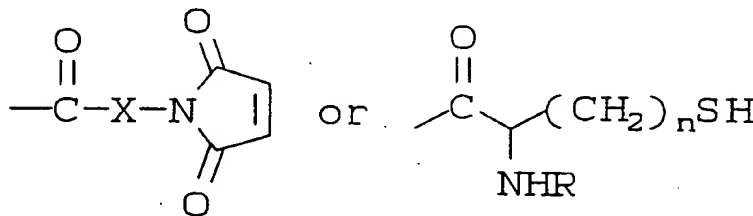
22. A compound having the formula

25

L-T

where T is a toxin and L is

30



- 48 -

where

X is C<sub>1</sub>-C<sub>5</sub> alkylene, phenyl, or C<sub>5</sub>-C<sub>6</sub>  
cycloalkylene;

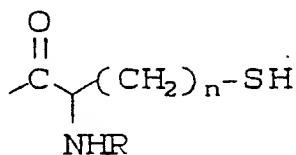
R is C<sub>1</sub>-C<sub>3</sub> alkanoyl; and

5 n is 1 or 2; and

23. A compound of Claim 22 where T is a  
Pseudomonas exotoxin.

10 24. A compound of Claim 23 where T is PE38M  
and L is

15

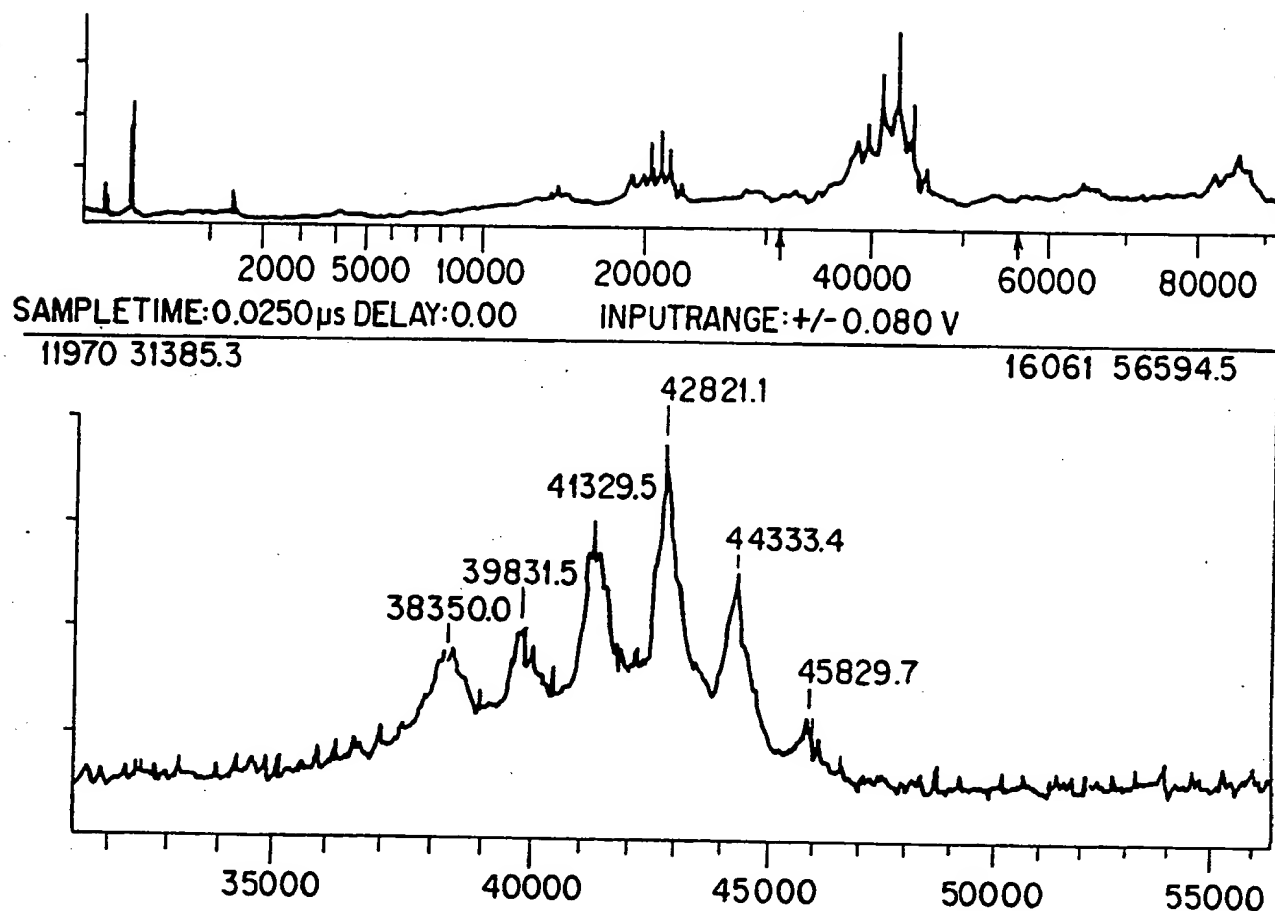


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MASS SPECTROMETRY  
LASER-TOF TECHNIQUE  
MATRIX ASSISTED MATRIX DESORPTION TOF MS  
MS OF CONJUGATE NewLys 38+ D-LYS6 GnRH

↓  
four Lys's  
1 to 5 GnRH's / PROTEIN

FIG. 1

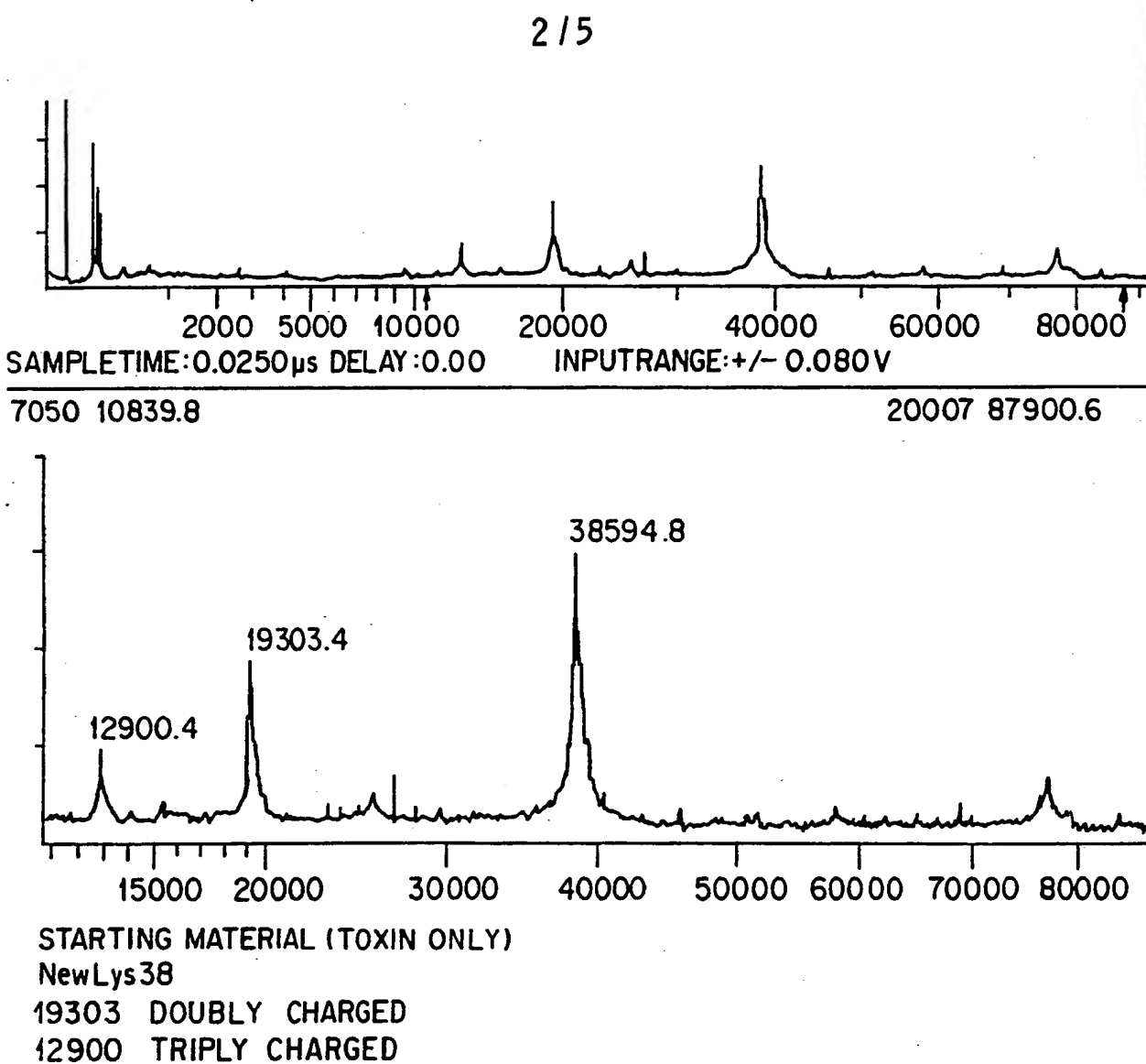


FIG. 2

SUBSTITUTE SHEET

## 12% Single Percentage Gel

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## 10% Single Percentage Gel

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## 12% Single Percentage Gel

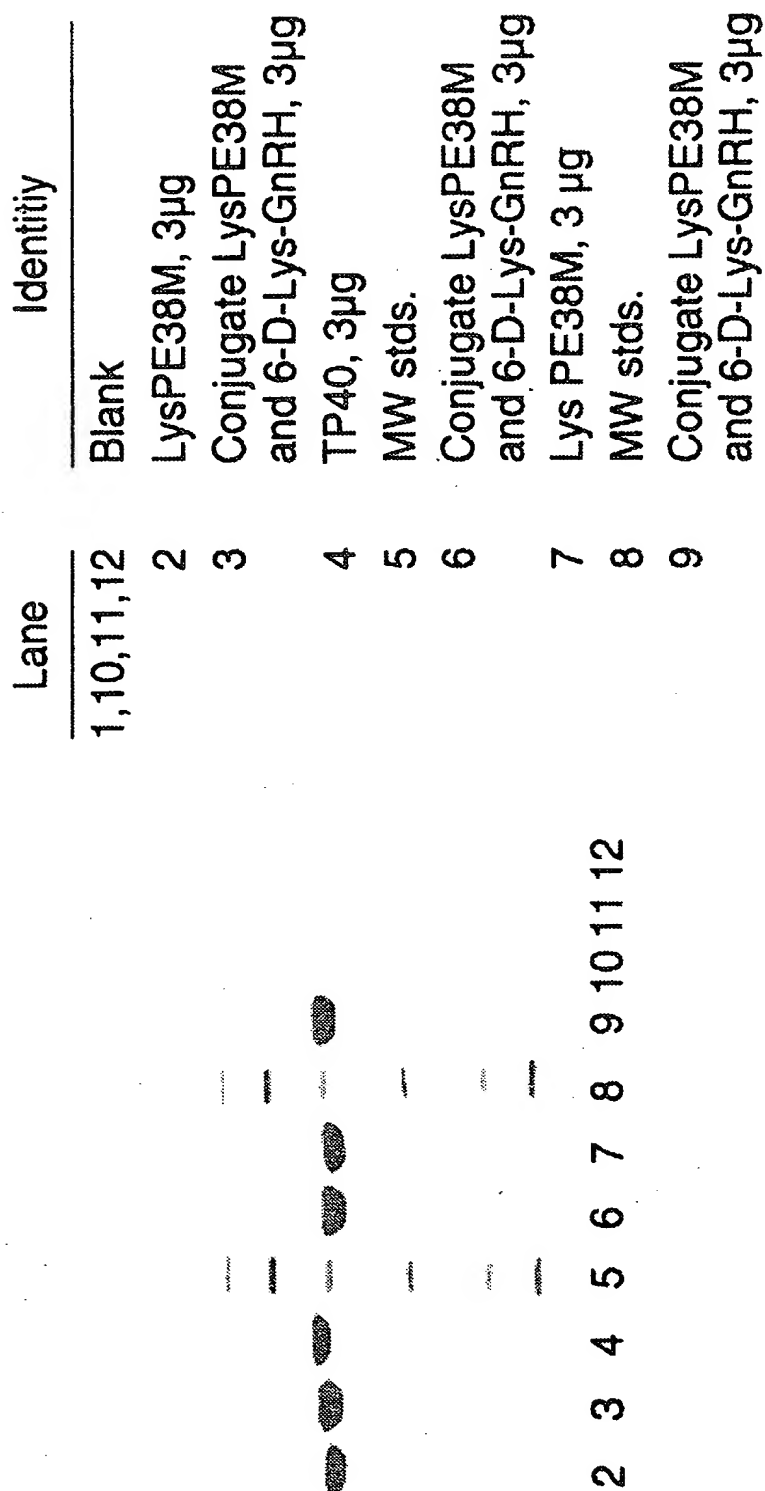


FIG. 5

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/01263

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 37/00, 37/02; C07K 5/00, 7/00, 15/00, 17/00

US CL : 530/328; 514/15

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/328; 514/15

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, CA

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 90/09799 (NETT ET AL.) 07 SEPTEMBER 1990, SEE ENTIRE DOCUMENT.	1-24
Y	US, A, 4,855,285 (STEVENS) 08 AUGUST 1989, SEE ENTIRE DOCUMENT.	1-24
Y	GB, A, 2,228,262 (TALWAR ET AL.) 22 AUGUST 1990, SEE ENTIRE DOCUMENT.	1-24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

27 April 1993

Date of mailing of the international search report

04 MAY 1993

Name and mailing address of the ISA/US  
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Washington, D.C. 20231

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